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These comments are submitted on behalf of the 750,000 members and supporters of People for the Ethical Treatment of Animals (PETA), in response to the NTP-CERHR's draft Expert Panel Reports on ethylene glycol and propylene glycol, prepared on December 5, 2002 (NTP-CENTER-EG-02 and NTP-CENTER-PG-02). PETA is the world's largest animal rights organization, and is committed to using the best available science to protect animals from suffering and to promote the acceptance of alternatives to activities that harm animals.

Our first, and most important, comment to make with respect to the two draft Reports is that in both cases the *Summaries, Conclusions and Critical Data Needs* section (Section 5.0) have not been completed, and the documents merely state that these are "To be completed during the Expert Panel meeting". It is difficult to see how PETA, or any other organization, can be expected to critique the planned work when we are given no indication as to what work is planned.

Bearing in mind the above proviso, we are submitting the following comments concerning hypothetical projected animal studies of human toxicity, and the reproductive and developmental toxicity in particular, of ethylene glycol and propylene glycol.

ETHYLENE GLYCOL

1. A large amount of animal data is already available

Ethylene glycol has been tested extensively on large numbers of various species of animals, for various types of toxicity. With respect to developmental and reproductive toxicity alone, more than 20 complete studies have been carried out, in mice, rats and rabbits (draft Report, pp. 51-104). In toxicokinetics, studies have also been carried out in monkeys, and even chimpanzees (draft, p. 26). None of the tests carried out have been validated, and conducting further non-validated animal tests will not result in additional relevant information.

Furthermore, optimal use has not been made of the available animal data. For example, the draft Report (p. 35, Lakind 1999) states that the mouse oral LD_{50} is 8.4-15.4 grams per kilogram of body weight (g/kg), yet the same document refers to a mouse study in which 12 g/kg, within the putative LD_{50} range, was administered daily for 2 years, with only minor toxicity (p. 41, NTP 1993). NTP-

CERHR needs to justify why the research requirement is now essentially for more of the same.

2. Interspecies variability

The toxicity of ethylene glycol varies widely between species, and animal data may therefore have little relevance for humans. In humans, the lethal dose is commonly stated to be 100 mL (1.1-2.2 g/kg for a body-weight range of 50-100 kg; EMBBS 1996), and a more precise estimate is that it is 1.4-1.6 g/kg (draft, p. 32), although most sources acknowledge a great deal of uncertainty with respect to this issue. In mouse studies, on the other hand, doses of up to 12 g/kg/day (an extraordinarily high dose, equivalent to approximately 1 kg/day for a man of average weight) were administered for up to 2 years, with the only effects being relatively minor hepatic and pulmonary toxicity (NTP 1993). Among non-human species that have been studied, even the lowest LD_{50} values, those of the rat and rabbit, at 4 mg/kg or more (Lakind 1999) and 5 mg/kg (Carney 1994), respectively, are several times higher than the human value.

As might be expected, the no-observed-adverse-effect level (NOAEL) for developmental toxicity also shows significant interspecies variability. The relationship between the NOAEL for fetal and maternal toxicity also shows such variability. In rats and mice, the maternal NOAEL (1.0 and at least 1.5 g/kg/day, respectively) is higher than the fetal NOAEL (0.5 and 0.15 g/kg/day, respectively), as is usual, whereas in rabbits the fetal NOAEL (2.0 g/kg/day) is higher than the maternal NOAEL (1.0 g/kg/day; draft, pp. 89-90).

In addition, it is probable that different mammalian species vary not merely in sensitivity to ethylene glycol, but in the mechanisms of its toxicity, because ethylene glycol toxicity is biochemically complex, and incompletely understood. The draft Report states: "the metabolic pathway of ethylene glycol … is qualitatively similar in humans, monkeys, dogs, rabbits, rats and mice" (p. 15). However, it is difficult to see any justification for such a sweeping statement, and the same Report mentions two studies that suggest a formaldehyde pathway for ethylene glycol metabolism in rats, which has never been found in other species (Kukielka 1991, Kadiiska 2000). Other differences between rats and rabbits in terms of ethylene glycol metabolism have also been found (p. 92). Even within the conventionally accepted human metabolism of ethylene glycol, there are several alternative metabolic pathways. Firstly, the metabolism of glycolaldehyde to glyoxylic acid can be via either glycolic acid or glyoxal. Secondly, there are at least five alternative pathways for the metabolism of glyoxylic acid, via glycine, oxalic acid, formic acid, hippuric acid, and benzoic acid. Furthermore, the alcohol dehydrogenase family of enzymes, which catalyze the first step in ethylene glycol metabolism, to glycolaldehyde, varies widely even between human races. There is also variability in the enzyme catalyzing the next step (aldehyde dehydrogenase), with approximately 50% of East Asians lacking one of the two isoenzymes (Pietrusczko 1980, Bosron 1986, Burnell 1989, Agarwal 2001).

The significance of the biochemical complexity and variability of ethylene glycol metabolism lies in the fact that ethylene glycol toxicity is due to a range of metabolites, and the roles of these have not been clearly defined. Unmetabolized ethylene glycol is only mildly toxic, its effect being ethanol-like central nervous system depression and mental state change. In an acute toxicity event, this mode of toxicity occurs the first after ingestion. The later modes of toxicity are then due to metabolites rather than to ethylene glycol itself:

(i) Glycolic acid gives rise to severe metabolic acidosis, characterized by low blood pH and bicarbonate level. Glyoxylic acid (a downstream metabolite) and lactic acid (formed from pyruvate concomitantly with the reduction of the nicotinamide adenine dinucleotide on which alcohol dehydrogenase is dependent) also contribute. The principal symptoms are cardiopulmonary, including Kussmaul respiration, cardiovascular palpitation, tachycardia, cyanosis, pulmonary edema and chest pain, with congestive heart failure in severe cases. Headache, visual disturbance, mental confusion, nausea, vomiting and diarrhea also occur.

(ii) If the oxalic acid pathway is followed, this leads to calcium oxalate precipitation in the blood, including the vascular tree, pulmonary parenchyma and myocardium, where it exacerbates the symptoms of metabolic acidosis, and may lead to congestive heart failure, pulmonary edema and pneumonitis. Later, precipitation in the renal cortex leads to decreased glomerular filtration and thus to renal insufficiency or failure. In addition, consumption of calcium by the formation of calcium oxalate may lead to severe hypocalcemia, the symptoms of which are often similar to those of acidosis.

(iii) Formic acid inhibits the cytochrome oxidase complex part of the respiratory process in the mitochondria, thus inhibiting oxidative phosphorylation and electron transport.

(iv) Neurotoxicity can occur at a late stage in ethylene glycol poisoning, but the cause of this is unclear.

There is no evidence that similar metabolic pathways are followed in different species, and this renders impossible interspecies extrapolation of the mechanism of toxicity. As one example, pyridoxine and thiamine are cofactors in the conversion of glyoxylic acid to glycine and _-hydroxy-ß-ketoadipic acid, so people (e.g. alcoholics) deficient in these vitamins produce more oxalic acid, and thus suffer more severe toxicity. Vitamin supplementation is therefore used to treat ethylene glycol poisoning (Hall 2002). However, vitamin nutrition differs widely between mammalian species. For example, some gastrointestinal bacteria synthesize thiamine, and species that digest these bacteria therefore usually obtain sufficient thiamine by this means, even on thiamine-deficient diets, whereas other mammals must obtain it from their diets, and are therefore more prone to thiamine deficiency (Subcommittee on Vitamin Tolerance 1987). The best-known group of gastrointestinal-bacteria-digesting mammals is the ruminants, which chew and then digest the contents of the rumenoreticulum after fermentation has taken place there. However, most herbivores and some omnivores have analogous, albeit less efficient, mechanisms for the gastrointestinal fermentation of plant material, and thus obtain thiamine by this route. For example, horses have fermentation in the cecum and colon, followed by absorption of nutrients through the epithelium. A number of other animals that have postgastric fermentation, such as rats, access its products by coprophagy, and rabbits perform a specialized type of coprophagy termed cecotrophy (Stevens 1995, Hirakawa 2001).

3. Exposure and epidemiological studies should have higher priority

Even were one to accept that animal studies provide valid data about human toxicity, they would not represent the type of research for which there is the most urgent requirement with respect to the human populations most at risk of chronic (including reproductive and developmental) toxicity, that is, those subject to occupational exposure. The occupational groups most at risk of chronic toxicity are workers responsible for de-icing aircraft, airport runways and highway bridges. Groups at lower risk are motor mechanics, and workers in polyester factories, etc. Two studies have shown that occupationally exposed populations have increased urinary ethylene glycol concentrations (Laitinen 1981, Gerin 1997). However, these studies were rather poorly designed, and included only small numbers of people (10 and 33), and the exposure levels are, as the draft Report admits, "not well characterized" (p. 9). Therefore, the first step should be to determine whether or not these populations do indeed have increased urinary and other levels of ethylene glycol and/or metabolites. Then, if the finding is positive, the next step should be to carry out a full epidemiological study.

Chronic exposure other than in the workplace is probably unimportant. Firstly, ethylene glycol has a half-life of only 0.2-0.9, 0.3-3.5, 2-12 and 8-48 days in soil, air, aerobic water and anaerobic water, respectively (draft, p. 3), with the final degradants being water and carbon dioxide. It is therefore extremely unlikely that people who live and work in areas without point-source ethylene glycol contamination will suffer significant chronic toxicity due to atmospheric or other environmental exposure. Exposure via drinking water or food is somewhat less improbable, but there are no reports of ethylene glycol in drinking water, and although it has been detected in wine and a number of foods, Health Canada estimates that the highest possible exposure is only in the _g/kg/day range (draft, p. 5).

4. In vitro *test methods are available*

If experimental studies are required after, or in addition to, epidemiological studies, the above-outlined potential for interspecies variability in the mechanism of action of toxicity means that the best approach to obtaining data about human ethylene glycol toxicity will be the use and/or development of *in vitro* test methods. With respect to embryotoxicity, one *in vitro* method that is already available is the rodent embryonic stem cell test, which has been validated by the European Centre for the Validation of Alternative Methods (Genschow 2002).

With respect to genotoxicity at least, the draft Report authors themselves appear to see *in vitro* data as more reliable than animal data, as two of three mammalian *in vivo* studies have shown ethylene glycol to be genotoxic, but the authors reject these findings on the ground that all *in vitro* studies have been negative (pp. 39-40).

5. Acute toxicity is the highest regulatory priority

Ethylene glycol has long been known to have high acute toxicity, responsible for a great deal of suffering and death. However, the practical and legislative response currently being made to this problem does not reflect its severity, so it is difficult to imagine effective responses being made to less severe types of toxicity, were reliable data about them to be obtained. Acute ethylene glycol toxicity is reported in approximately 5,000 human cases in the USA per year (Litovitz 2001), and resulted in 29 deaths in 1994, together with a far greater number of people suffering irreversible damage to the kidneys, heart, lungs, and/or central nervous system. The overwhelming majority of

these poisoning events involve antifreeze, most brands of which contain 85-95% ethylene glycol. The causes of these poisonings are manifold, including suicide and murder attempts; people who are drunk mistaking antifreeze for an alcoholic drink; confusions over drink containers in mechanics' workshops; and young children mistaking antifreeze for a soft drink (it has a sweet taste). The majority of the deaths are of young children.

Pets, especially dogs, also frequently suffer antifreeze poisoning, for the same reason that children do, and in 2001 at least 107 dogs died of this cause in California (Donatelli 2002), equivalent to nearly 900 nationwide. This is probably only a fraction of the actual number of deaths, as many are not reported, if the animals die quickly, or if the owners are too poor or uncaring to contact a veterinary surgeon, for example. It is also probable that large numbers of wild animals die from drinking antifreeze spilt on roads. There are no data for this, but in 1992 an endangered California condor died of this cause (Los Angeles Zoo 2002), and deaths of pumas and raccoons have also been recorded ("Antifreeze poisoning in a raccoon" 1993).

The majority of deaths due to acute antifreeze toxicity could be prevented by either of two simple measures. Firstly, a bittering agent (e.g. denatonium benzoate) could be added, to discourage people and animals from ingesting more than a minimal quantity. This was mandated in California on September 27, 2002, and adds only approximately 3 cents per gallon to the cost of antifreeze (Bill No. AB 2474 2002, Donatelli 2002). Secondly, ethylene glycol could be replaced with propylene glycol in many of its applications, including antifreeze. A number of companies already manufacture propylene glycol-based antifreeze. The toxicity of propylene glycol is discussed below, but at this point it is sufficient to stress that it is far lower than that of ethylene glycol.

6. Simple measures for reducing chronic human exposure are readily available

The exposure of at-risk populations could readily be reduced by certain simple measures. Furthermore, these measures are inherently advisable even if the exposed people do not in fact suffer ethylene glycol toxicity. Firstly, most of the workers in the studies mentioned used little or no protective equipment, and the use of such equipment should always be encouraged when people are in regular contact with any aerosol. Secondly, replacement of ethylene glycol with propylene glycol, as suggested with respect to acute toxicity, would probably have a positive effect on hypothetical chronic as well as on acute toxicity. Thirdly, it is possible that the addition of a bittering agent, also as suggested with respect to acute toxicity, would, for example, encourage workers to wash their hands more thoroughly before eating. It is not frivolous to suggest that the demand for more data may be driven by a desire actually to avoid action and/or legislation that would be likely to reduce risks to humans and animals.

PROPYLENE GLYCOL

Propylene glycol has long been considered to be more or less safe for humans, except at very high doses, and the FDA places it in the category "generally recognized as safe" (21 CFR 184.1666 2002). There are therefore few restrictions on its use, and it is more or less freely added to food, materials in contact with food (e.g. cellophane wraps), tobacco products, medicines, cosmetics, etc. In Japan, the mean dietary intake has been estimated to be 0.7 mg/kg/day (Louekari 1990). Propylene glycol is also used in numerous industrial processes, as well as household and industrial products. However, a certain reassessment of its non-toxicity has taken place over the past decade, and this is probably connected with the NTP's decision to assess its reproductive and developmental toxicity.

Part of the reassessment has been concerned with the putative non-toxicity of propylene glycol as a pharmaceutical excipient. The reassessment has led to acknowledgment that propylene glycol is toxic at high doses. It has also become clear that certain populations are especially sensitive to propylene glycol toxicity. The most sensitive populations are infants (draft, p. 41), pregnant women, people with renal or hepatic failure (draft, p. 40), and patients treated with disulfiram or metronidazole. In addition, women, East Asians, Native Americans and Eskimos are moderately sensitive. Some drugs containing propylene glycol, such as Agenerase, a protease inhibitor for treatment of HIV-1 infection, must therefore now be distributed with contraindications and/or warnings with respect to these populations (Glaxo 2000).

The reassessment has also been propelled by the US Federal Trade Commission's 1995 ruling that propylene glycol-based antifreeze must not be marketed as "safe", "non-toxic" and "environmentally safer", and must not be contrasted in these respects with ethylene glycol-based antifreeze (FTC 1995). The reasons for the Federal Trade Commission's ruling were twofold:

(i) Propylene glycol is not absolutely safe to humans or animals if drunk in large volumes. Containers must therefore now bear the following legend: "Cautionary Information: This product may be harmful if swallowed. Store safely away from children and pets. Do not store in open or unlabeled containers".

(ii) The claim that propylene glycol is environmentally safer than ethylene glycol is disputable. On the one hand, both compounds are rapidly degraded and are neither biopersistent nor bioaccumulative, so are relatively harmless to the environment as a whole. On the other hand, antifreeze tends to become contaminated with lead and other heavy metals when used in engines, so used antifreezes of either type are approximately equally environmentally damaging, if they enter watercourses, for example. There was some concern that presentation of propylene glycol-based antifreeze as environmentally friendly would encourage people to dispose of it more carelessly. Propylene glycol's superiority over ethylene glycol rests solely on its much lower acute toxicity. However, as wild animals, including members of rare species such as condors and pumas, are sometimes poisoned by ethylene glycol-based antifreeze, propylene glycol can in this sense be considered more environmentally friendly.

Concern about the possible chronic, reproductive and developmental toxicity of propylene glycol is certainly legitimate. The most important point to stress is that the current use of this compound offers an almost ideal situation for human epidemiological studies, as some people

may have little exposure, most people have moderate chronic exposure, from food, etc., and certain occupational groups (e.g. motor mechanics, aircraft de-icing workers) are subject to heavy exposure. <u>However, no epidemiological studies have been carried out.</u> Even tissue exposure has barely been investigated, with only one limited study having been conducted, in just 10 motor mechanics. In this study, the urinary propylene glycol levels of men exposed to this compound were not found to increase (Laitinen 1995). The first priority should be to carry out exposure studies. Then, if the results warrant concern, a complete, large-scale epidemiological study should be carried out.

If experimental studies are required in addition to epidemiological studies, the rational approach would be to carry these out *in vitro* rather than *in vivo*. A considerable amount of work on the development of *in vitro* methods for assessing propylene glycol toxicity has already been carried out (Morshed 1994, 1998), but none of this information was mentioned in the draft Report. In addition, as mentioned above, a validated *in vitro* embryotoxicity method is available (Genschow 2002).

Further animal studies are inappropriate for assessing propylene glycol toxicity for the following reasons:

1. A large amount of animal data is already available

Propylene glycol has been tested extensively on large numbers of various species of animals, for various types of toxicity. With respect to developmental and reproductive toxicity alone, seven complete studies have been carried out, in mice, rats, hamsters, rabbits, domestic fowl and *Hydra* (draft, pp. 46-61). For other types of toxicity, studies have also been carried out on various other species, including cats (draft, p. 18), dogs (draft, p. 29), and monkeys (draft, pp. 32-33). A number of studies that have been carried out are not mentioned in the draft Report (Morshed 1995). As none of the tests carried out have been validated, conducting further non-validated animal tests will not result in additional relevant information.

2. *Interspecies variability*

The toxicity of propylene glycol shows a considerable amount of interspecies variability (draft, p. 31). In addition, the toxicity shows a great deal of intraspecies variability, with LD_{50} values in rats, for example, ranging between 8 and 46 g/kg (draft, p. 27).

More importantly than merely the difference in sensitivity, it is probable that different mammalian species vary in the mechanisms of toxicity of propylene glycol, because the elimination rates and metabolic pathways vary. As with ethylene glycol, the toxicity mechanism of the unmetabolized alcohol is probably similar to that of ethanol (draft, pp. 13, 44). However, in humans, more of the compound is excreted in the unmetabolized form than is the case with ethylene glycol. The serum half-lives of the two glycols are probably similar, at 2-4 hours for propylene glycol (Speth 1987) and, disputably, 2.5-8.4 hours for ethylene glycol (EG draft Report, p. 31), so the higher proportion of the unmetabolized form of propylene glycol being excreted is probably largely due to less being metabolized. This may contribute to the much lower toxicity of propylene glycol (less being converted into more toxic metabolites), and may also be responsible for the apparently greater contribution of ethanol-type toxicity to the overall toxicity of propylene glycol. The proportion excreted in the unmetabolized form shows marked interspecies variability, being 45% in humans

(Arbour 2000), but up to 88% in dogs (Ruddick 1972), and 14-24% in rabbits (Yu 1987), and this in itself (leaving aside whatever difference in metabolism is responsible for this difference) is likely to mean that the contribution of ethanol-type toxicity to overall toxicity varies widely between species. As with ethylene glycol, the likelihood of interspecies variability is supported by the high level of genetic polymorphism of alcohol and aldehyde dehydrogenases even between human races (Pietrusczko 1980, Bosron 1986, Burnell 1989, Agarwal 2001).

It is probable that the principal additional mechanism of propylene glycol toxicity is metabolic acidosis, caused mainly by the metabolite lactic acid (draft, p. 13-14). Lactic acid occupies a position in propylene glycol metabolism analogous to that occupied by glycolic acid in ethylene glycol metabolism (an aldehyde, lactaldehyde in this case, is the intermediate), and, as with ethylene glycol, there is an alternative pathway, via methylglyoxal (analogous to glyoxal). However, the pathways are more complex in the case of propylene glycol than in the case of ethylene glycol, and are no more clearly understood. Merely with respect to the above upstream portion of the metabolism, the lactic acid formed directly from lactaldehyde contains both stereoisomers, whereas the lactic acid formed by the methylglyoxal pathway consists only of the D-form. In addition, there is a third pathway to lactic acid, by phosphorylation followed by dephosphorylation. The relative importance of these pathways influences the severity of toxicity, as both stereoisomers give rise to the same level of acidosis but L-lactic acid is metabolized more rapidly than D-lactic acid, and is more prone to be used in gluconeogenesis (draft, p. 14). Methylglyoxal synthetase activity is induced by ketosis, resulting in a higher proportion of the D-form in the lactic acid, and thus increasing the probability of metabolic acidosis as a result of propylene glycol absorption (draft, p. 14). Ketosis occurs in people who are starving, diabetic, or on diets with abnormally high protein plus fat to carbohydrate ratios, but it is more readily induced in some species than in others, being particularly common in ungulates, and the etiology of ketosis is highly complex, occurring when animals are either overfed or underfed, and with the criteria for these conditions varying between species. Furthermore, propylene glycol does not exacerbate ketosis in all species, but is actually used to treat it in sheep and goats (Aiello 1998). This is just one area in which the mechanism of propylene glycol toxicity shows marked interspecies variability.

Stereospecificity also shows interspecies variability, at least with respect to the phosphorylation-dephosphorylation pathway to lactic acid, as rabbits metabolize the L-form of propylene glycol more readily, whereas rats and mice metabolize both stereoisomers equally well (Huff 1961, Miller 1965). Stereospecificity in the other pathways may also show interspecies variability, as in horses and rabbits alcohol dehydrogenase oxidizes L-propylene glycol more readily than D-propylene glycol, but this difference has not been shown in other species (Huff 1961). The draft Report authors admit that "it is not possible to piece together a complete picture of stereospecific metabolism of D,L-propylene glycol" (draft, p. 15). This admission is without interspecies variability being taken into consideration.

In humans, most or all of the propylene glycol that is neither excreted in the unmetabolized form nor converted, by whichever pathway, to lactic acid, is conjugated with glucuronic acid and then excreted in the urine. However, felids (members of the cat family) lack the ability to form glucuronic conjugates (Lakind 1999), and the cat study referred to in the draft Report is therefore of little value (pp. 18-19). Furthermore, felids are not unique in this respect, but are at the end of a continuum of effectiveness of glucuronidation, with mustelids, for example, glucuronidating more than felids but less than the few other species that have been studied. To make matters more

complicated, the cause of non-glucuronidation is different in felids and mustelids, and, in addition, mustelids show a sexual difference in this respect (Court 2001). Defective glucuronidation is probably connected with an obligately carnivorous diet, and thus lack of exposure to phytoalexins (Court 2000), but the correlation between carnivory and non-glucuronidation cannot be assumed, as dogs appear to have markedly high rates of glucuronidation. Humans are in the middle of the range, but the effect on propylene glycol toxicity is unclear (Court 1997). The draft Report does not propose the species in which to carry out tests, and we are therefore unable to make any more detailed criticism on this issue.

A third mechanism of toxicity, which has been little investigated, is hemolysis, due to the osmolality gap (draft, p. 13). There may also be other mechanisms. Any step in any metabolic pathway may show interspecies variability, analogous to cats' inability to form glucuronic conjugates, which cannot be predicted and might only be found by chance. The relevance of such possibilities to reproductive and developmental toxicity is unknown, as the two principal known mechanisms are mainly relevant to acute toxicity. Overall, it is difficult to see how the authors can be sufficiently confident to state that "much is known about the mechanism of action" (draft, p. 13), "The metabolism of propylene glycol is well understood" (draft, p. 13), and "The database is sufficient to understand and predict metabolic clearance of D,L-propylene glycol in man" (draft, p. 16).

A final point with respect to propylene glycol toxicity is that, as with ethylene glycol, the exposure of at-risk populations could readily be reduced by increased use of protective equipment, which should in any case always be encouraged when workers are in regular contact with any aerosol.

Finally, from the point of view of the ethics of animal experimentation, it must be mentioned that the authors describe a report of a range of acute and subchronic studies in rats and rabbits as "enjoyable reading" (draft, p. 29). This sentiment seems incompatible with the supposed widespread acceptance of the principle of the 3Rs (reduce, refine, replace) with respect to animal experimentation, not to mention the lip service given to reducing the suffering of animals. We are therefore unconvinced that serious consideration will be given to the non-necessity of the additional studies that may be proposed.

Yours sincerely,

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